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Abstract
In Chlamydomonas reinhardtii and other green algae, a pathway which actively concentrates CO2 at the site of ribulose 1,5-bisphosphate-carbozylase/oxygenase (RUBISCO) is responsible for the suppression of photo-respiration and oxygen inhibition of photosynthesis and for the stimulation of photosynthesis at a low external CO2 concentrations. Increased photosynthesis and reduced photorepiration in Chlamydomonas at air levels of CO2 and O2 are manifested by a high affinity for CO2 in photosynthesis, a nearly maximal photosynthetic rate, absence of O2 inhibition of CO2 fixation, low rates of synthesis of photo respiratory metabolites, and a near-zero CO2 compensation concentration (1,2). The CO2-concentrating pathway of Chlamydomonas is inducible, with induction occurring at air levels of CO2 but not at elevated (1-5%) concentrations of CO2 (2). Biochemical and physiological studies implicated the involvement of at least two components in the pathway, an energy-dependent, saturable inorganic transport process (1,9) and the enzyme carbonic anhydrase (CA) (1,2). Badger et al. (1) suggested that the role of CA in the pathway might be dehydration of transported HCO3 to supple CO2, the substrate of RUBISCO. In order to further characterize the Chlamydomonas CO2-concentrating pathway, we utilized existing, nonphotosynthetic mutants of C. rienhardtii fo study of induction requirements and set out to identify and characterize new mutant strains of C. reinhardtii with defects in the CO2-concentrating pathway itself. This work with Chlamydomonas mutants has helped firmly establish the requirement for photosynthetic competence in the induction of the pathway, unambiguously confirmed that at least two components, CA and HCO3 transport, are involved, and that the principal role of internal CA is dehydration of transported HCO3.

Disciplines
Botany

Comments
This chapter is published as Spalding, MH, RJ Spreitzer, WL Ogren 1985 Use of mutants in analysis of the CO2-concentrating pathway of Chlamydomonas reinhardtii. In W Lucas, J Berry, eds ., Inorganic Carbon Uptake by Photosynthetic Organisms, American Society of Plant Physiologists, Rockville, Maryland. pp. 361-375. Posted with permission.

Inorganic Carbon Uptake by Aquatic Photosynthetic Organisms WJ Lucas and JA Berry Eds

CHAPTER 23

USE OF MUTANTS IN ANALYSIS OF THE CO_2-CONCENTRATING PATHWAY OF *CHLAMYDOMONAS REINHARDTII*

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INTRODUCTION

In *Chlamydomonas reinhardtii* and other green algae, a pathway which actively concentrates CO_2 at the site of ribulose 1,5-bisphosphate-carboxylase/oxygenase (RUBISCO) is responsible for the suppression of photorespiration and oxygen inhibition of photosynthesis and for the stimulation of photosynthesis at low external CO_2 concentrations. Increased photosynthesis and reduced photorespiration in *Chlamydomonas* at air levels of CO_2 and O_2 are manifested by a high affinity for CO_2 in photosynthesis, a nearly maximal photosynthetic rate, absence of O_2 inhibition of CO_2 fixation, low rates of synthesis of photorespiratory metabolites, and a near-zero CO_2 compensation concentration (1, 2).

The CO_2-concentrating pathway of *Chlamydomonas* is inducible, with induction occurring at air levels of CO_2 but not at elevated (1-5%) concentrations of CO_2 (2). Biochemical and physiological studies implicated the involvement of at least two components in the pathway, an energy-dependent, saturable inorganic transport process (1, 9) and the enzyme carbonic anhydrase (CA) (1, 2). Badger *et al.* (1) suggested that the role of CA in the pathway might be dehydration of transported HCO_3^- to supply CO_2, the substrate of RUBISCO.

In order to further characterize the *Chlamydomonas* CO_2-concentrating pathway, we utilized existing, nonphotosynthetic mutants of *C. reinhardtii* for study of induction requirements and set out to identify and characterize new mutant strains of *C. reinhardtii* with defects in the CO_2-concentrating pathway itself. This work with *Chlamydomonas* mutants has helped firmly establish the requirement for photosynthetic competence in the induction of the pathway, unambiguously confirmed that at least two components, CA and HCO_3^- transport, are involved, and that the principal role of internal CA is dehydration of transported HCO_3^-.

MATERIALS AND METHODS

All methods have been described previously (8-12). The wild-type *C. reinhardtii* strain utilized was 2137 mt+(15). The cell-wall-less strain CW 15 mt+ and mutant strain F60 mt− were obtained from Dr. R. Togasaki. Mutant strains rcl-u-1-10-6c, NLS 6-2A, 11-4D, 12-5A, and 12-7 are nonphotosynthetic mutants derived from 2137 mt+ (14-16). Strain CC424 (CW-15 arg-2 sr-u-2-60 mt−) was obtained from the Duke *Chlamydomonas* culture collection.

RESULTS AND DISCUSSION

Induction of the CO_2-concentrating pathway.

Photosynthesis-deficient mutants of *C. reinhardtii* have been used to study the regulation of the CO_2-concentrating pathway in this alga. With wild-type *Chlamydomonas*, induction of the pathway occurred at air levels of CO_2 and O_2 (0.03% and 21%, respectively) in the light. Induction did not occur in the dark nor in the light when the CO_2 concentration was raised to 5%, and induction was depressed by exposure to low O_2 concentrations (8). Lack of induction of the pathway in the dark and inhibition of induction of CA by the photosynthetic-electron-transport inhibitor DCMU (13) indicated that photosynthesis may be involved in the regulation of the CO_2-concentrating pathway. A requirement for photosynthesis in the induction of CA activity was confirmed by the observation that photosynthesis-deficient mutants of *C. reinhardtii* lacked substantial CA activity when exposed to inducing conditions (Table I; refs. 8, 13). As illustrated in Table I (see also 8) photosynthesis-deficient *Chlamydomonas* mutants also lack the capacity to actively accumulate DIC. It appears that photosynthetic competence is required for induction of both CA activity and the capacity for active DIC accumulation. Further, the apparent requirements of low

CO_2 and high O_2 concentrations for induction implicate photorespiratory metabolism in the induction process.

Table I. Carbonic Anhydrase Activity and DIC Concentrating Capacity in Photosynthesis-Deficient Mutants of *Chlamydomonas reinhardtii* Exposed to Inducing Conditions.

C. reinhardtii Strains	CA[a] (units mg Chl^{-1})	DIC_i[b] (mM)
wild type (2137)	521	2.5
F60[c]	62	0.6
rcl-u-10-6*c*[d]	97	–
NLS 6-2A[e]	102	0.5
11-4D[f]	36	0.6
12-5A[g]	109	–
12-7[h]	88	0.4

[a]Assay method and unit definition as in (8).

[b]Maximal internal DIC concentration during a 120 s time course. Initial external DIC concentration was 80 μM, at pH 7.0.

[c]Acetate requiring mutant defective in phosphoribulokinase (6).

[d]Light sensitive, acetate requiring mutant (*rcl-u*-1 locus) defective in the large subunit of RUBISCO (14).

[e]Same as (d) but with a mutation which suppresses light sensitivity (16).

[f]Light sensitive, acetate requiring mutant deficient in PS II activity (15).

[g]Light sensitive, acetate requiring mutant deficient in photophosphorylation (7,15).

[h]Light sensitive, acetate requiring mutant deficient in PS I activity (15).

Identification of mutants defective in the CO_2-concentrating pathway.

The CO_2-concentrating pathway allows *Chlamydomonas* to maintain photosynthetic rates near the maximum at air levels of CO_2 and O_2 because of the greatly increased internal CO_2 concentration (1). Since cells grown at 5% CO_2 (CO_2-concentrating pathway not induced) have very low rates of photosynthesis at air levels of CO_2 and O_2, it was reasoned that mutants unable to actively concentrate CO_2 internally would grow poorly, if at all, at air levels of CO_2 and O_2. At 5% CO_2, however, such mutants should be able to grow nearly as well as the wild type, since CO_2 would no longer be limiting. A preliminary experiment

showed that wild-type cells exposed to the CA inhibitor ethoxyzolamide (EZ) at a concentration of 150 μM grew very poorly at air levels of CO_2, but were indistinguishable from control wild-type cells if grown at 5% CO_2 (data not shown). Since CA was thought to be an important component of the pathway, these results suggested that mutants probably would have the expected phenotype.

Following mutagenesis with ethylmethanesulfonate, the mutagenized population of 2137 mt+ was screened for mutants which grew poorly relative to wild type at air levels of CO_2, but grew as rapidly as wild type at 5% CO_2. Approximately 25 strains exhibiting this phenotype were recovered. The growth rates of several of these mutant strains relative to wild type are indicated in Table II, along with physiological characteristics of some of them. There is obvious variation in both phenotypes and physiological characteristics, suggesting that this collection of mutants might be rich in information regarding the CO_2-concentrating pathway of *Chlamydomonas*. Two of the mutant strains, *ca*-1-12-1C and *pmp*-1-16-5K, have been analyzed in more detail and are discussed below.

Table II. Relative Growth Rates and Some Physiological Characteristics of *Chlamydomonas reinhardtii* Mutants Requiring Elevated CO_2 Concentrations for Photoautotrophic Growth.

Strain	Growth		[a]Photosynthesis	$\Gamma(\mu l \cdot l^{-1})$		CA
	air	5%CO_2	(rel.)	2%O_2	50%O_2	(units mg Chl^{-1})
wt (2137)	+++	+++	100	< 10	< 10	620
ca-1-12-1C	+	+++	16	15	210	130
pmp-1-16-5K	−	+++	25	< 10	< 10	480
15-2P	−	++	ND[b]	< 10	< 10	430
15-7F	−	+	ND	ND	110	105
15-2ZY	+	++	56	< 10	< 10	230
16-1B	−	+	ND	< 10	< 10	ND
18-1D	−	+	ND	< 10	< 10	480
18-2A	−	+	ND	ND	ND	530
18-6A	−	++	ND	> 350	> 350	ND
18-7C	−	++	ND	ND	> 350	ND

[a]CO_2-dependent O_2 evolution with 80 μM external DIC, at pH 7.0; values are percent relative to wildtype.
[b]Not determined.

Mutant with reduced CA activity.

One of the mutants analyzed, *ca*-1-12-1C, was found to have reduced CA activity relative to wild type. The CA activity in this mutant was initially reported to not increase substantially following induction of the

CO_2-concentrating pathway (10). It has become apparent from further work that CA activity in this mutant does increase during inducing conditions but is substantially reduced relative to wild type (Table III). The reason for the apparent discrepancy between our initial characterization and more recent work is not clear at this time. However, all other characteristics of this mutant, as discussed below, remain unchanged.

Table III. Distribution of CA Activity in Four Strains of *Chlamydomonas reinhardtii.*

C. reinhardtii strains conditions	CA (units mg Chl^{-1})		
	Medium[a]	Total[b]	External[c]
Wild type (2137):			
5% CO_2	ND[d]	34 ± 17	31 ± 12
2 days air	ND	1991 ± 164	842 ± 69
ca-1-12-1C:			
5% CO_2	ND	21 ± 15	20 ± 13
2 days air	ND	492 ± 120	325 ± 101
CW 15:			
5% CO_2	211 ± 93	15 ± 8	17 ± 9
2 days air	$14,508 \pm 671$	87 ± 5	35 ± 4
CW 15 *ca*-1[e]:			
5% CO_2	125 ± 74	12 ± 10	11 ± 8
2 days air	$5,570 \pm 429$	53 ± 6	49 ± 6

[a]Activity remaining in supernatant after centrifuging to remove the cells. Based on chlorophyll concentration of the suspension prior to centrifugation.

[b]Assayed after exposing the cells to 0.2% nonidet P-40 (nonionic detergent) at 38°C for 10 min.

[c]Assayed in well-washed, intact cells.

[d]Not detectable.

[e]Cell-wall-less strain carrying the *ca*-1 mutation. Recovered from a cross between *ca*-1-12-1C (mt+) and CC 424 (mt−).

Although total activity of CA is reduced in the *ca*-1-12-1C mutant relative to wild type, the activity apparently 'external' to the cell (3) appears to be less affected than the portion of the activity not assayable in intact cells (i.e. difference between total and external activities in Table III). In fact, there was no significant difference found between total and external CA activities in a cell-wall-less *ca*-1 mutant. These data suggest that *ca*-1-12-1C is specifically or preferentially deficient in an internal

CA. As mentioned earlier, the CO_2-concentrating pathway is responsible for wild-type *C. reinhardtii* having a near-maximal photosynthetic rate and exhibiting little or no O_2 inhibition of photosynthesis at air levels of CO_2 and O_2, and having a near-zero, O_2-insensitive CO_2 compensation concentration. These properties are illustrated by the data presented in Table IV along with similar data for EZ-inhibited wild type and some mutants. In contrast to wild type, the *ca*-1 mutant had a photosynthetic rate only 15% of maximum at 350 $\mu l\ l^{-1}$ CO_2 and low O_2 even though the maximum photosynthetic rate of the mutant was similar to wild type. The photosynthetic rate of the *ca*-1 mutant was also very O_2 sensitive, and it exhibited a very high, O_2-sensitive CO_2 compensation concentration. The characteristics of EZ-inhibited wild type were found to be very similar to those of the *ca*-1 mutant, lending support to the conclusion that this mutant is deficient in CA.

Table IV. Physiological Characteristics of *Chlamydomonas reinhardtii* Wild Type and Mutant Strains following 2 days of Inducing Conditions.

Characteristic	Condition	Strains				
		wt	+EZ[a]	*ca*-1	*pmp*-1	*ca pmp*
Photosynthetic rate (μmoles CO_2 or O_2 mg Chl^{-1} h^{-1}):						
350 $\mu l\ l^{-1}$ CO_2[b]	2%O_2	142	40	28	66	28
	21%O_2	145	25	14	37	15
	50%O_2	141	19	4	21	9
Urating Sat. CO_2[c],	21%O_2	199	204	179	171	173
CO_2 compensation concentration ($\mu l\ l^{-1}$):						
	2%O_2	<10	30	19	<10	23
	21%O_2	<10	45	81	<10	48
	50%O_2	<10	87	244	<10	225
Internal DIC[d] (mM):		2.7	14.0	12.5	0.5	4.5

[a] 50μM ethoxyzolamide.
[b] Rates measured by infrared gas analysis.
[c] Rates measured as CO_2-dependent O_2 evolution in an O_2 electrode with 2.5 mM $NaHCO_3$, pH 7.0.
[d] Maximum value measured during a 120 s time course, 80 μM external DIC (initial concentration) at pH 7.0.

The low photosynthetic rates and O_2 inhibition of CO_2 fixation observed in the *ca*-1 mutant at 350 $\mu l\ l^{-1}$ CO_2, but a photosynthetic rate similar to wild type at high CO_2 indicated that photosynthesis, in this strain, was severely CO_2-limited at 350$\mu l\ l^{-1}$ CO_2. This conclusion was strongly supported by analysis of labelled products following photosynthesis at either low (100 μM $NaHCO_3$, pH7.0) or high (2.5mM

$NaHCO_3$, pH7.0) CO_2 concentrations in the presence of 21% O_2 (Table V). In wild type, only 5% of the incorporated label was found in photorespiratory metabolites (glycolate, glycine, serine) at either CO_2 concentration. However, in the *ca*-1 strain, 46% of the label was located in these metabolites at 100 μM $NaHCO_3$, but only 9% at 2.5 mM $NaHCO_3$. Partitioning of carbon between photosynthesis and photorespiration is determined by the relative CO_2/O_2 concentration ratio at the site of RUBISCO (4). Thus, at 100 μM $NaHCO_3$, the internal CO_2 concentration of the *ca*-1 mutant must have been much lower than that of wild type. At 2.5 mM $NaHCO_3$ both wild type and the *ca*-1 mutant exhibited similar product profiles, indicating that no significant difference exists between these two strains at a saturating CO_2 concentration.

Table V. Products of Photosynthetic $^{14}CO_2$ Fixation by *Chlamydomonas reinhardtii* Wild Type and Mutant Strains Following 2 days of Inducing Conditions.

$NaHCO_3$ Concentration	Fraction	% of Total ^{14}C-products[a]			
		wt	*ca*-1	*pmp*-1	*ca pmp*
100μM	insoluble	50	18	6	25
	glycolate	1	35	6	10
	glycine	2	9	12	8
	serine	2	2	5	4
2.5 mM	insoluble	40	30	46	47
	glycolate	0	2	1	1
	glycine	2	3	3	1
	serine	3	4	3	2

[a]5 min at pH 7.0, 21% O_2.

Data on accumulation of internal DIC (Table IV) indicated that the *ca*-1 mutant accumulated DIC internally to a concentration nearly five times higher than that observed in wild type. Wild type treated with 50 μM EZ accumulated DIC to an internal concentration similar to the *ca*-1 strain. Since the *ca*-1 mutant (and EZ-inhibited wild type) was demonstrated to be strongly CO_2-limited under similar conditions, one can not escape the conclusion that the accumulated DIC in the *ca*-1 mutant (and EZ-inhibited wild type) must be HCO_3^- and that this HCO_3^- must not be undergoing dehydration rapidly enough to supply CO_2 (substrate for RUBISCO) for photosynthesis. This would be the case only if HCO_3^- was transported, either into the cell or into the chloroplast, and CA was not available in the mutant to catalyze the dehydration of the transported

HCO_3^-. Even with an internal concentration of 15mM, the uncatalyzed dehydration of HCO_3^- would supply CO_2 at a rate of only approximately 7 μmol mg Chl^{-1} h^{-1} (Spalding and Portis, unpublished results). The characteristics of the *ca*-1 mutant, combined with the observation that the CA inhibitor EZ duplicated in wild-type *Chlamydomonas* the physiological and biochemical manifestations of the lesion in the *ca*-1 strain, provide overwhelming evidence that the *ca*-1 mutant is deficient in CA activity in the internal compartment into which HCO_3^- is transported.

Mutant with reduced inorganic carbon transport.

The second mutant strain characterized, *pmp*-1-16-5K (11), exhibited a photosynthetic rate at 350 $\mu l\ l^{-1}$ CO_2 which was very low relative to wild type and was inhibited by O_2, but achieved a maximum photosynthetic rate similar to that of wild type (Table IV). In these characteristics the *pmp*-1 mutant was similar to the *ca*-1 mutant and was apparently also strongly CO_2-limited at 350 $\mu l\ l^{-1}$ CO_2. However, the *pmp*-1 mutant differed from the *ca*-1 strain in that CA activity was similar to wild type (11) and exhibited a low CO_2 compensation concentration at all O_2 concentrations tested (Table IV). In addition, this mutant accumulated very little DIC internally (Table IV), no more in fact than could be accounted for by passive accumulation by wild-type cells in the dark (11). Thus, while photosynthesis in the *pmp*-1 mutant was strongly CO_2-limited at air levels of CO_2, as in the *ca*-1 mutant, the CO_2 limitation appeared to be due to an inability to actively transport DIC at rates sufficient to maintain high rates of photosynthesis. Although this mutant appeared to be deficient in DIC transport capacity, this activity was not completely absent. Treatment of the mutant cells with 50 μM EZ blocked dehydration of transported HCO_3^- and allowed HCO_3^- to accumulate as in wild type, but to a lesser extent than wild type (11). This was interpreted as indicating that the capacity for HCO_3^- transport in the *pmp*-1 mutant was reduced, but not totally absent.

The low, apparently O_2-insensitive CO_2 compensation concentration of the *pmp*-1 mutant (Table IV) is somewhat difficult to explain. However, a computer simulation of the CO_2 assimilation system of *Chlamydomonas* demonstrated that it is possible for a mutant with reduced transport to maintain a low CO_2 compensation concentration even though it was strongly CO_2-limited at air levels of CO_2 (Spalding and Portis, unpublished results).

The distribution of photosynthetic products in the *pmp*-1 mutant at low CO_2 showed an increase in labelling of photorespiratory metabolites relative to that observed with wild type, as was found with the *ca*-1 mutant (Table V). However, the labelling profile differed from that of

the *ca*-1 mutant in that much less label accumulated in glycolate. The reason for this difference in labelling patterns between the *ca*-1 and *pmp*-1 mutants still remains unclear. In the presence of saturating CO_2 (2.5 mM $NaHCO_3$, pH7.0) the labelling pattern for the *pmp*-1 mutant was also similar to that of wild type, indicating no major difference in the CO_2 assimilation of the mutant at a saturating CO_2 concentration (Table V).

Mutant with reduced CA and DIC transport activities.

In order to further demonstrate that the *ca*-1 and *pmp*-1 mutations were distinct and to evaluate the interaction of these two mutations, a double mutant was constructed genetically and its characteristics determined (12). As expected, photosynthesis at 350 $\mu l\ l^{-1}$ CO_2 was markedly reduced relative to wild type and was inhibited by O_2 (Table IV), and the level of CA activity was low, similar to that of the *ca*-1 mutant (12). The level of accumulation of internal DIC was elevated relative to wild type or the *pmp*-1 mutant (Table IV) and was similar to that observed with the *pmp*-1 mutant treated with EZ (11). Thus, the internal DIC accumulation observed in the double mutant was consistent with a deficiency of CA activity in the compartment into which HCO_3^- is transported and a reduction in the capacity for HCO_3^- transport.

The elevated, O_2-sensitive CO_2 compensation concentration observed in the double mutant (Table IV), similar to that of the *ca*-1 mutant, would also be expected if the *pmp*-1 lesion resulted in an incomplete block of the pathway (retention of some transport capacity) and the *ca*-1 lesion resulted in a complete or nearly complete block of the pathway at a point following the *pmp*-1 lesion in the pathway. In other words, the real 'bottle-neck' in the CO_2-concentrating pathway, in both the *ca*-1 mutant and the double mutant, lies at the same point in the pathway, dehydration of HCO_3^- to supply CO_2 for RUBISCO. Thus, the *ca*-1 mutant and the double mutant would be expected to exhibit similar physiological characteristics, except that DIC accumulation would be expected to be somewhat lower in the double mutant, as was observed. Distribution of photosynthetic products at 100 μM $NaHCO_3$ in the double mutant indicated increased photorespiratory activity relative to wild type, but the labelling pattern appeared intermediate between those of the *ca*-1 and the *pmp*-1 mutant (Table V). Although we speculated previously that the increased glycolate labelling observed in the *ca*-1 mutant may reflect excretion to offset the large accumulation of HCO_3^- (12), it is still unclear whether this or some other factor is responsible for the differences in photosynthetic labelling patterns observed with these three mutant strains.

CO_2-Exchange characteristics during dark-light transitions.

The CO_2-exchange characteristics of wild-type *C. reinhardtii* in the presence or absence of the CA inhibitor, EZ, are illustrated in Fig. 1A. In the absence of the inhibitor, the cells exhibited a nearly square-form change from CO_2 efflux to CO_2 uptake on the transition from dark to light and the reverse upon the transition from light to dark. In the presence of EZ, a burst of CO_2 uptake was observed during the first 3-4 min after the dark to light transition, followed by a reduced rate of uptake often so extreme that net CO_2 efflux occurred. Following this efflux the net rate of CO_2 uptake rose, with minor oscillations, to a steady state level roughly ten-fold lower than the uninhibited rate. Following light to dark transitions, a marked post-illumination burst of CO_2 efflux was observed in the presence of EZ before a steady-state rate of dark CO_2 efflux was attained.

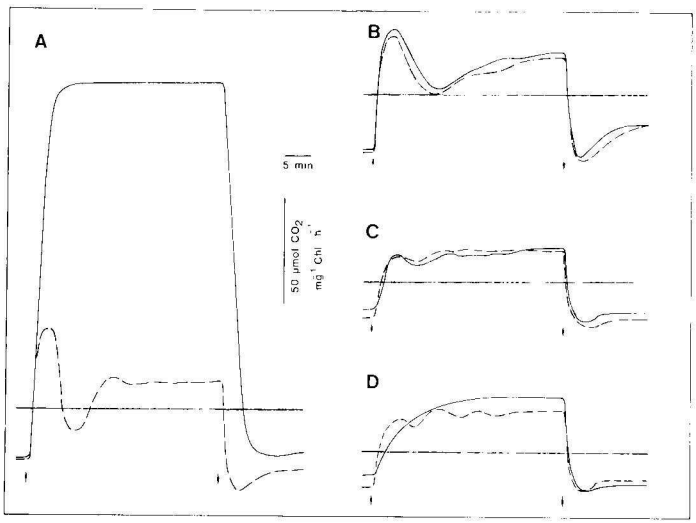

Figure 1. CO_2-exchange patterns of A) wild type (2137 *mt+*), B) CA deficient (*ca*-1-12-1C), C) DIC transport deficient (*pmp*-1-16-5K), and D) double mutant (*ca pmp*) *Chlamydomonas reinhardtii*. Patterns were recorded either with (dashed line) or without (solid line) the addition of 50 μM EZ in the dark 5 min prior to illumination. CO_2 exchange was monitored at 25°C, with 350 $\mu l\ l^{-1}$ CO_2 and 21% O_2. Arrows indicate light on (↑) and light off (↓), and zero CO_2 exchange is indicated by the light horizontal line.

The CO_2-exchange characteristics of the *ca*-1 mutant in both the presence and absence of EZ were similar to those of wild type inhibited with EZ (Fig. 1B). These CO_2-exchange characteristics were unaffected by the addition of excess CA (data not shown), demonstrating that the observed pattern of CO_2-exchange was not due to disequilibration between CO_2 in the gas and aqueous phases.

Figure 1D illustrates the CO_2-exchange characteristics of the *pmp*-1 mutant. Oscillations were apparent with the EZ-inhibited cells but were not as dramatic as with wild-type cells (cf. Fig. 1A). Inhibition of the steady-state photosynthetic rate by EZ was also less pronounced with the transport deficient mutant than with wild type, but the rate of photosynthesis in the presence of EZ was similar for both wild type and mutant cells. The post-illumination CO_2 efflux was also increased in the EZ-inhibited cells of the *pmp*-1 mutant.

As with the *ca*-1 mutant, the CO_2-exchange characteristics of the double mutant (*ca pmp*) were essentially unaffected by EZ (Fig. 1C). The CO_2-exchange pattern of the double mutant was very similar to that of the EZ-inhibited *pmp*-1 mutant (Fig. 1D). Since the *ca*-1 mutation results in a deficiency of internal CA activity, the combination of this mutation with the *pmp*-1 mutation in the double mutant would be expected to behave similarly to the *pmp*-1 mutant with CA inhibited.

In order to better understand the nature of the burst of CO_2 uptake observed upon illumination in the *ca*-1 mutant and EZ-inhibited wild type, and to a lesser extent in the double mutant and EZ-inhibited *pmp*-1 mutant, $^{14}CO_2$ uptake into both inorganic and fixed carbon was monitored under conditions similar to those of the CO_2-exchange measurements. In uninhibited wild-type cells, both total ^{14}C uptake and incorporation of ^{14}C into fixed carbon increased linearly with time (Fig. 2A) as might be expected from the CO_2-exchange pattern (Fig. 1A). Internal DIC increased from less than 500 μM in the dark to 2-4 mM in the light, then dropped immediately back to the dark level when the light was removed (Fig. 2A).

In the presence of EZ, however, the total uptake of $^{14}CO_2$ into the wild-type cells increased rapidly upon illumination, very similar to that observed with CO_2-exchange. This burst of CO_2 uptake was apparently due entirely to uptake into an internal DIC pool, as incorporation of ^{14}C into fixed carbon proceeded at a much lower, nearly linear rate in the light. The internal DIC concentration increased to about 25 mM during the first 3-4 min of illumination, but the concentration subsequently dropped to a steady-state light level of approximately 10 mM. This steady state level of internal DIC is similar to the level observed previously in short-term experiments (Table IV). It is apparent that

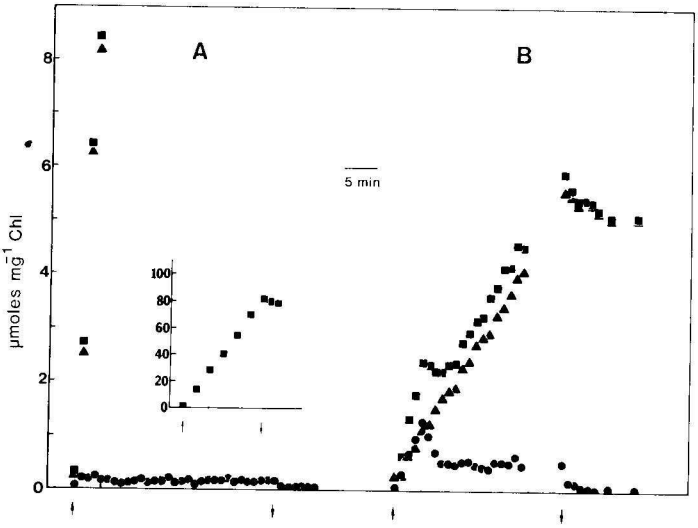

Figure 2. Uptake of $^{14}CO_2$ into inorganic (•), fixed (▲), and total (■) carbon pools in wild type *C. reinhardtii* either in the absence (A) or in the presence (B) of 50 μM EZ. Other conditions as indicated for Fig. 1. Arrows indicate light on (↑) and light off (↓). Inset (A) indicates time course on a larger scale of total carbon uptake in the absence of EZ.

DIC was released from the cell during the drop in internal concentration from 4-7 min after illumination, since the total ^{14}C content of the cells actually decreased even though fixation of ^{14}C continued at a relatively steady rate. This release of DIC from the cells corresponds to the net CO_2 efflux observed in the CO_2-exchange measurements at about 5 min after illumination (Fig. 1A).

After the light to dark transition, the high internal DIC pool of the EZ-inhibited wild-type cells returned to the dark level (less than 500 μM) within about 3 min (Fig. 2B). The amount of fixed ^{14}C also decreased when illumination was discontinued, but this was almost entirely accounted for by excretion of fixed carbon into the medium (data not shown). These observations indicate that the post-illumination burst of CO_2 efflux, observed in the CO_2-exchange experiments (Fig. 1A) was due primarily to release of the internal DIC pool rather than release of newly-fixed carbon (photorespiration). The characteristics of $^{14}CO_2$ incorporation and distribution in the *ca*-1 mutant were similar to those of the EZ-inhibited wild type (data not shown).

It is apparent that a deficiency in internal CA leads to excessive ac-

cumulation of internal DIC upon illumination, and that the resulting state of disequilibrium is relieved by excretion of this excess HCO_3^-. The mechanism responsible for these observations is unknown. In the presence of CA, HCO_3^- is rapidly dehydrated to CO_2 and OH^-. In the absence of CA, HCO_3^- accumulates and OH^- is not released. Since the OH^- is thought to be the anion excreted in a loosely coupled exchange for HCO_3^- (5), lack of HCO_3^- dehydration could cause a charge imbalance, perhaps leading to an extreme membrane hyperpolarization which could drive the efflux of HCO_3^-.

Considerably less HCO_3^- accumulated in the EZ-inhibited *pmp*-1 mutant and the double mutant, indicated by a much smaller burst of CO_2 uptake upon illumination than observed in EZ-inhibited wild type and the *ca*-1 mutant (Fig. 1). These observations are consistent with the *pmp*-1 mutation causing a reduction in HCO_3^- transport activity, since a reduced rate of HCO_3^- transport may permit the cell to react before the HCO_3^- overshoot became very extreme.

Following the initial rapid uptake and excretion of DIC, the cells pass through a series of increasingly dampened oscillations in DIC influx (Fig. 1). These oscillations may represent progress by the cell in achieving equilibrium between the rates of HCO_3^- uptake, HCO_3^- dehydration, and CO_2 fixation, but again the details of the mechanism which regulates the response are presently unkown.

SUMMARY

The use of photosynthesis-deficient mutants of *C. reinhardtii*, in conjunction with manipulation of growth conditions for wild type *Chlamydomonas*, made it possible to demonstrate that induction of the CO_2-concentrating pathway requires photosynthesis and probably requires photorespiratory metabolism as well. Since the *ca*-1, *pmp*-1, and *ca pmp* mutants and other 'CO_2-requiring' mutants exhibit photorespiratory metabolism under higher CO_2 concentrations than wild-type *C. reinhardtii*, it may be possible to learn more about the requirements for induction using these mutants. Mutants blocked in the photorespiratory pathway would also prove useful in this regard.

Dysfunctions in the *C. reinhardtii* CO_2-concentrating pathway lead to a reduced CO_2 concentration at the site of RUBISCO, so CO_2 assimilation by this alga acquires some properties of higher plant C_3 photosynthesis. These properties include a much reduced photosynthetic rate at air concentrations of CO_2 and O_2, inhibition of photosynthesis by O_2, and synthesis of photorespiratory metabolites at low CO_2 concentrations in the presence of O_2. Reduced internal CA also leads

to an accumulation of a greatly elevated concentration of internal DIC. Since the substrate of RUBISCO is CO_2 rather than HCO_3^-, the *ca*-1 mutant accumulates a high concentration of HCO_3^-, but photosynthesis is, nonetheless, CO_2-limited because the uncatalyzed dehydration of HCO_3^- to CO_2 occurs at a rate insufficient to saturate photosynthesis. In the absence of internal CA activity, internal DIC concentrations undergo marked oscillations during dark to light transitions, suggesting that CA might also be necessary to supply OH^- for counter exchange during HCO_3^- transport. Thus, internal CA is an essential component of the CO_2-concentrating pathway of *C. reinhardtii.*

In the *pmp*-1 mutant, photosynthesis is again CO_2-limited because this mutant appears unable to transport DIC into the cell at a rate commensurate with maximum photosynthetic rate. Thus, this undefined transport process is a second essential component of the CO_2-concentrating pathway. Most of the additional 'CO_2-requiring' mutants so far isolated are not allelic to either the *ca*-1 or the *pmp*-1 loci, indicating additional components are probably required for a fully operational CO_2-concentrating pathway in *Chlamydomonas.* Analysis of the remaining approximately 20 'CO_2 requiring' mutants and identification and analysis of more such mutants should prove very useful in understanding this CO_2-concentrating pathway.

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